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The apoplastic pool of abscisic acid in cotton leaves in relation to stomatal closure

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Abstract. Suboptimal nitrogen nutrition, leaf aging, and prior exposure to water stress all increased stomatal closure in excised cotton (*Gossypium hirsutum* L.) leaves supplied abscisic acid (ABA) through the transpiration stream. The effects of water stress and N stress were partially reversed by simultaneous application of kinetin (N^6-furfurylaminopurine) with the ABA, but the effect of leaf aging was not. These enhanced responses to ABA could have resulted either from altered rates of ABA release from symplast to apoplast, or from some "post-release" effect involving ABA transport to, or detection by, the guard cells. Excised leaves were preloaded with [^{14}C]ABA and subjected to overpressures in a pressure chamber to isolate apoplastic solutes in the exudate. Small quantities of ^{14}C were released into the exudate, with the amount increasing greatly with increasing pressure. Over the range of pressures from 1 to 2.5 MPa, ABA in the exudate contained about 70% of the total ^{14}C, and a compound co-chromatographing with phaseic acid contained over half of the remainder. At a low balancing pressure (1 MPa), release of ^{14}C into the exudate was increased by N stress, prior water stress, and leaf aging. Kinetin did not affect ^{14}C release in leaves of any age, N status, or water status. Distribution of ABA between pools can account in part for the effects of water stress, N stress, and leaf age on stomatal behavior, but in the cases of water stress and N stress there are additional kinetin-reversible effects, presumably at the guard cells.

Key words: Abscisic acid and stomata – Cytokinin and stomata – *Gossypium* (ABA, stomata) – Leaf aging – Nitrogen nutrition and stomata – Phaseic acid – Stoma (ABA and kinetin effects) – Water stress.

Abbreviations and symbols: ABA = abscisic acid; PA = phaseic acid; ψ_w = water potential

Introduction

It has long been accepted that abscisic acid (ABA) mediates stomatal closure during periods of water stress (reviewed by Raschke 1975a). However, evidence has accumulated that stomatal closure in intact stressed leaves can precede increases in bulk levels of tissue ABA (Beardsell and Cohen 1975; Walton et al. 1977; Henson 1982; Cornish and Zeevaart 1985). Two explanations have been advanced. First, leaf ABA may consist of active (apoplastic) and inactive (symplastic) pools, with varying ratios between the two. Considerable indirect evidence (see Walton 1980) and some direct evidence (Cornish and Zeevaart 1985) support this viewpoint, and a mechanism to regulate distribution between the two pools has been proposed (Cowan et al. 1982). Second, sensitivity of the target cells, i.e. the guard cells, to ABA in the apoplastic pool may vary (Trewavas 1981). This argument receives support from reports that water stress increases stomatal response to ABA applied to isolated epidermis (Wilson 1981) and intact or excised leaves (Davies 1978; Ackerson 1980). Changes in responsiveness to ABA may be mediated by other growth substances such as a cytokinin (Cooper et al. 1972; Das et al. 1976; Blackman and Davies 1983, 1984a) or an auxin (Snaith and Mansfield 1982).

Stomatal responses to water stress and to ABA are strongly increased in N-stressed leaves (Radin and Ackerson 1981; Radin et al. 1982) and during the aging of leaves after they have reached full expansion (Jordan et al. 1975; Ackerson 1980; Radin 1981; Blackman and Davies 1984b). Again, the apparent sensitization to ABA could result either from altered partitioning of leaf ABA between pools, or from altered responses of the guard cells. Here we report studies of the effects of water stress, nitrogen deficiency, and leaf age on stomata of

cotton, using a recently-developed technique to sample the apoplastic pool of ABA (Ackerson 1982; Cornish and Zeevaart 1985). The technique involves pressure-induced exudation of apoplastic sap from excised leaves.

Material and methods

Plant material. Cotton plants (*Gossypium hirsutum* L. cv. Deltapine 70; Delta and Pine Land Co., Casa Grande, Ariz., USA)[a] were grown from seed in a glasshouse as described in Radin et al. (1982). Pots were watered three times weekly with nutrient solution containing (in mmol·l^{-1}): $Ca(NO_3)_2$, 2; KNO_3, 1; KH_2PO_4, 0.5; $MgSO_4$, 1; NaCl, 0.5; Fe-ethylenediaminetetraacetate, 0.045; H_3BO_3, 0.04; $MnSO_4$, 0.01; $ZnSO_4$, 0.001; Na_2MoO_4, 0.0004; and $CuSO_4$, 0.0002. Deficiencies of N were established by substituting $CaCl_2$ and KCl for 80% of the $Ca(NO_3)_2$ and KNO_3, respectively. Leaves were studied at three stages of development: during expansion (blade ≤ 4 cm wide), after cessation of expansion (youngest fully expanded leaf), or after aging (four nodes below the youngest fully expanded leaf). In most cases, the aged leaves were located at node 1, the first node above the cotyledons, and the youngest expanded leaves were at node 5. In some experiments, expanding and recently expanded leaves were also at node 1, but on younger plants. The effect of aging was independent of position on the plant.

When plants had developed fully expanded leaves at node 5, water stress was initiated by withholding water from the pots. Water potentials at midday were monitored with a pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, Cal., USA). The leaf excised for ψ_w determination was several nodes below the most recently expanded leaf.

Stomatal responses to ABA. Leaves were generally treated as described in Radin et al. (1982). When tests involved an expanding leaf, plants were placed in a growth chamber in darkness at 30° C, and all leaves except the test leaf were removed. The stems were severed under water and the trimmed stems were transferred to test tubes. A portion of the stem was left attached because the petioles were too short to reach the bottom of suitably sized test tubes. When test leaves were fully expanded, they were excised with petioles under water, and the excised leaves were handled as above. Large leaves were trimmed to about 100 cm^2. Stock solutions of kinetin (N^6-furfurylaminopurine) and ABA (both from Sigma Chemical Co., St. Louis, Mo., USA) were added to the tubes to obtain the desired concentrations, then the lights were turned on to initiate stomatal opening. Explants were left in the light (570 μmol photons·m^{-2}·s^{-1} between 400 and 700 nm) 3 h before measurement of diffusive conductances; the reported values are the sums of abaxial and adaxial conductances. Both photon flux and conductances were determined with a LI-1600 steady-state porometer (LiCor Instruments, Lincoln, Neb., USA) fitted with a LI-190B quantum sensor. Conductances are reported as functions of ABA concentration rather than dose (concentration × volume of water transpired). Under the conditions of these tests, transpiration was only slightly affected by the differences in leaf conductance (Radin et al. 1982).

[a] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Stock solutions of ABA were made by dissolving the ABA in a small volume of ethanol or methanol, then diluting it to 10 μM with distilled water. Neither alcohol by itself, at the levels used, had any effect upon stomatal behavior. Kinetin was dissolved in water (final concentration of stock 100 μM) with extensive stirring in a boiling water bath.

Partitioning of [^{14}C]ABA between pools. Leaves were excised, trimmed as before if appropriate, and put into small test tubes, and the volume of water in the tube was adjusted to 1.9 ml. To this was added 3.8 kBq of [G-^{14}C](±)-ABA in 0.1 ml aqueous solution (947 MBq mmol^{-1}; Amersham Corp., Arlington Heights, Ill., USA). The final concentration of ABA was 2 μM. In some cases the uptake solution also contained 10 μM kinetin. After the lights were turned on, leaves were incubated at 25° C for approx. 2 h, at which time the excess [^{14}C]ABA solution was removed and replaced with an identical solution containing unlabelled ABA. The leaves remained in this unlabelled "chase" solution for 1 h. Leaf water potentials remained high (≥ -0.4 MPa) during these manipulations.

At the end of the [^{14}C]ABA pulse and [^{12}C]ABA chase periods, each leaf was removed from its solution, and the portion of the petiole which had been immersed in the solution was excised. The leaf was placed in the pressure chamber, and a small tube was fitted to the cut end of the petiole to carry exuded sap into a collection vial. The pressure chamber was lined with moist paper to maintain humidity during pressurization. The pressure was slowly increased to 1.0 MPa with N_2 or air and maintained at that level until equilibration of the leaf (cessation of flow). During exudation, gas escaping through the cut end of the petiole kept the tube from filling with solution. Thus, when collection at 1 MPa was ended, the volume of exudate remaining in the delivery tube was very low (0–2 μl). The tube was removed, rinsed with a small volume of methanol, and this rinse was combined with the collected exudate. After replacement of the tube on the petiole, the pressure was raised to 1.5, 2.0, or 2.5 MPa and the process was repeated. Equilibration at each pressure required 30–45 min. Each leaf was incubated at 1 MPa and only one higher pressure; thus for any leaf the total time in the pressure chamber did not exceed 75 min. Pressurization with air instead of N_2 did not alter the results.

After release of the pressure, the leaf was removed from the chamber and its blade area determined with a LiCor LI-3000 leaf-area meter (LiCor Instruments). Five discs of total area 1.40 cm^2 were removed from the leaf blade (avoiding major veins), frozen at −80° C, and combusted to $^{14}CO_2$ in a tissue oxidizer (R.J. Harvey Instruments, Hillsdale, N.J., USA). The $^{14}CO_2$ was trapped in scintillation fluid containing phenylethylamine (Woeller 1961) and its radioactivity determined in a scintillation counter. Exudates were mixed with Ready-Solv MP scintillation fluid (Beckman Instruments, Fullerton, Cal., USA) and counted directly. All counts were corrected for quenching and background. The leaf blades were also frozen at −80° C, then lyophilized for later analysis of ABA.

Analyses of leaves and exudates. Abscisic acid was extracted from the lyophilized leaves by a slight modification of the procedure of Guinn and Brummett (1987). Dichloromethane was substituted for diethyl ether in the partitioning, and an additional purification step employing a silica Sep-Pak cartridge (Waters Associates, Milford, Mass., USA) was included prior to high-performance liquid chromatography (HPLC). Tracer quantities of [^{3}H]ABA (Amersham) were added during the initial extraction step to allow calculation of recoveries. Exudates had many fewer contaminants than leaf blades, and purification

was simpler. Exudates were extracted with hexane containing 10 mg·l^{-1} butylated hydroxytoluene, then acidified by adding an equal volume of 5 mM HCl. The aqueous fraction was partitioned five times against ethyl acetate. The ethyl-acetate fractions were combined and dried at 40° C under N_2, and the residue was redissolved in a small volume of methanol. This was injected, with water bracketing (Guinn and Hendrix 1985), onto a C_{18} HPLC column and eluted with 50% methanol containing 0.02 M acetic acid. Eluted compounds were detected by absorption at 254 mm and quantitated by peak heights. Retention times of ABA and phaseic acid (PA) were established by HPLC of similarly treated standards. Eluted compounds were collected in scintillation vials, mixed with scintillation fluid, and their radioactivity determined in the scintillation counter.

Solute release during pressurization. Leaves were pressurized as above except that exudates were not mixed with methanol. The first 10 µl of exudate volume was discarded. Exudates collected thereafter were diluted appropriately with glass-distilled water and electrical conductivities determined with a Wheatstone bridge (Yellow Springs Instruments, Yellow Springs, O., USA) equipped with a standard 1-cm conductivity cell. Conductivities were compared to a standard curve constructed using KNO_3 solutions, and KNO_3 concentration equivalents were calculated for the original, undiluted extracts.

Results

Nitrogen deficiency and leaf aging both increased the degree of stomatal closure after application of ABA through the transpiration stream (Fig. 1). In expanding or recently expanded leaves of unstressed plants, ABA at concentrations up to 1 µM had minimal effect, but aging significantly increased the response (Fig. 1 A). With 1 µM ABA, conductance of expanding or recently expanded leaves was decreased only about 25%, but conductance of old leaves was decreased 50%. In N-stressed plants, stomata responded more strongly to applied ABA at all three stages, again with a large increase in response in the aging leaves (Fig. 1 B). With 1 µM ABA, conductance was decreased approx. 50% at the expanding and recently expanded stages, and 75% when leaves were old.

The comparisons in Fig. 1 A and B are based upon leaves all at node 1 on plants of different ages. When leaf 1 was old, node 5 held the youngest fully expanded leaf. Stomatal responses to ABA in high-N and low-N leaves at this node were similar to those of leaves at node 1 when they had recently expanded (Fig. 1 C). Thus, further comparisons of young and old leaves refer to the most recently expanded blade and the blade four nodes below it (at nodes 5 and 1, respectively). This procedure allowed simultaneous harvest of young and old leaves from the same plants.

The concentration of exogenous ABA causing 5% stomatal closure (incipient closure as defined

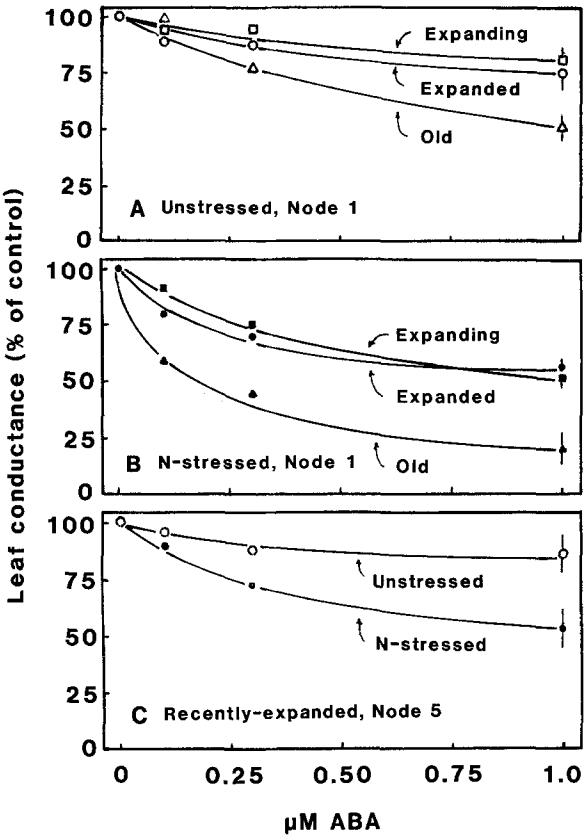

Fig. 1A–C. Effect of ABA on stomatal conductance of excised cotton leaves of three ages and two N levels. All leaves were presented ABA through the transpiration stream for 3 h before measurement of conductance. For each curve, values are the means of three separate experiments. Representative SEs are shown at 1 µM ABA

by Raschke 1975b) was estimated by interpolation from the data in Fig. 1. It varied from about 0.01 µM for old N-stressed leaves (the most sensitive) to 0.08 µM for young unstressed leaves (the least sensitive). These concentrations correspond to doses of approx. 0.1–0.8 pmol·cm^{-2} of (+)-ABA, the active isomer, transported into the leaf. These sensitivities are similar to threshold doses found by others (Raschke 1975b; Mansfield and Davies 1983).

Kinetin and ABA interacted differently in leaves of different ages and N levels. In young unstressed leaves, neither compound had a significant effect on conductance at the concentrations applied (Table 1). In old unstressed leaves, response to ABA was much greater, as found earlier, but kinetin still had little effect. In contrast, in N-stressed leaves of both ages kinetin characteristically partially reversed the effect of ABA (Table 1). Thus, kinetin appeared to cause stomata of N-stressed and high-N leaves to behave similarly. An

Table 1. Stomatal conductances of excised cotton leaves. Abscisic acid and kinetin were supplied through the transpiration stream at 1 μM and 10 μM, respectively. For each treatment, conductances are expressed as a percentage of the water-only control. Conductances of controls were as follows (in $cm \cdot s^{-1}$): Young, unstressed, 1.12±0.07; old, unstressed, 0.82±0.09; young, N-stressed, 1.00±0.07; old, N-stressed, 0.69±0.15; young, water-stressed, 1.20±0.11. Each entry in the table is the mean±SE from four to fourteen experiments. The 95% confidence limits are approximately twice the SE

Leaf age	Stress treatment	Stomatal conductance (% of control)		
		ABA	Kinetin	ABA+Kinetin
Young	Unstressed	87±11	102± 6	85± 7
Old	Unstressed	40± 6	95±20	39± 5
Young	N stress	49± 8	102± 9	75± 8
Old	N stress	28± 4	126±13	59±17
Young	Water stress	21± 4	97±12	58±12

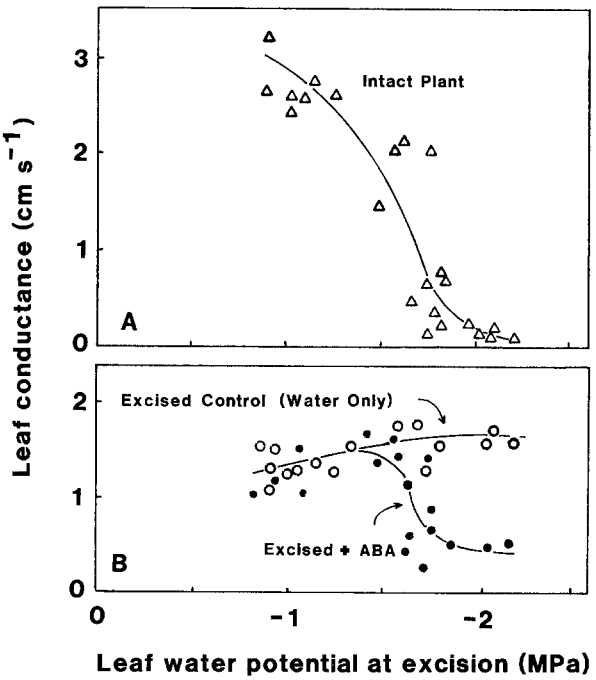

Fig. 2A, B. Effect of water stress on stomatal conductance of leaves of intact cotton plants (**A**) and subsequent stomatal response to ABA in excised leaves (**B**). The ABA was fed through the transpiration stream. Each point represents a single leaf

effect of aging, which was not reversible by kinetin, was superimposed upon the effect of N deficiency.

Allowing high-N plants to become water-stressed before leaf excision had effects similar to those of N stress. Stomata of the intact leaves closed with declining ψ_w, reaching a conductance approx. 50% of the initial at a ψ_w of −1.6 MPa (Fig. 2A). When leaves were excised during the stress cycle, stomata of excised leaves were generally unresponsive to ABA when the ψ_w before excision was higher than −1.6 MPa. After that time they became highly responsive to ABA (Fig. 2B). This effect of water stress was noted despite the fact that the tests necessarily involved relief of the stress, i.e., the leaves were fully hydrated during ABA uptake. As with N stress, the effects of water stress were partially reversible by kinetin (Table 1).

The failure of kinetin to affect old unstressed leaves (Table 1) implies that leaf aging increased stomatal response to ABA by a different mechanism from that of water stress or N stress. Partitioning of ABA was deemed one possible basis for this difference (although differential catabolism of the kinetin was also possible). Our methods for studying partitioning involved feeding [^{14}C]ABA, then following the accumulation of label in the apoplastic sap. Of the ^{14}C that appeared in the exudates over the range of 1−2.5 MPa applied pressure, a mean of 71% cochromatographed with ABA (Fig. 3A). Of the remainder of the label, 60% cochromatographed with PA, the first metabolite of ABA (Walton 1980). No other individual labeled compound was identified. Both the ^{14}C and ABA contents of the exudate increased with increasing pressure (Fig. 3A, B) but the total ABA content of the leaf did not (Fig. 3C). Presumably the induction time at high pressure was inadequate to initiate extensive accumulation of ABA, as two previous studies indicated a lag time greater than 1 h (Ackerson 1982; Ackerson and Radin 1983). Interestingly, the PA content of the exudates also increased rapidly with increasing pressure (Fig. 3B), indicating that its partitioning may have been governed by similar factors to those governing ABA partitioning. Although PA was the major ^{14}C-labeled compound other than ABA in the exudates, its close correlation with ABA meant that release of ^{14}C was still a good indicator of the release of ABA. When ^{14}C and ABA contents of exudates were expressed as percentages of the totals in the leaf, they were linearly related with a correlation coefficient of 0.90 (Fig. 3, insert). Thus the release of ^{14}C provided a fast and reasonably accurate indication of the release of ABA from these leaves over a range of pressures from 1 to 2.5 MPa.

The effects of N stress, water stress, and leaf aging on ^{14}C release were determined at a pressure of 1.0 MPa, too low to dehydrate the leaves to zero turgor (Radin and Parker 1979). Exudate from young unstressed leaves contained 0.14% of the total ^{14}C of the leaf (Table 2). This value was increased to 0.37% for the N-stressed leaves. At

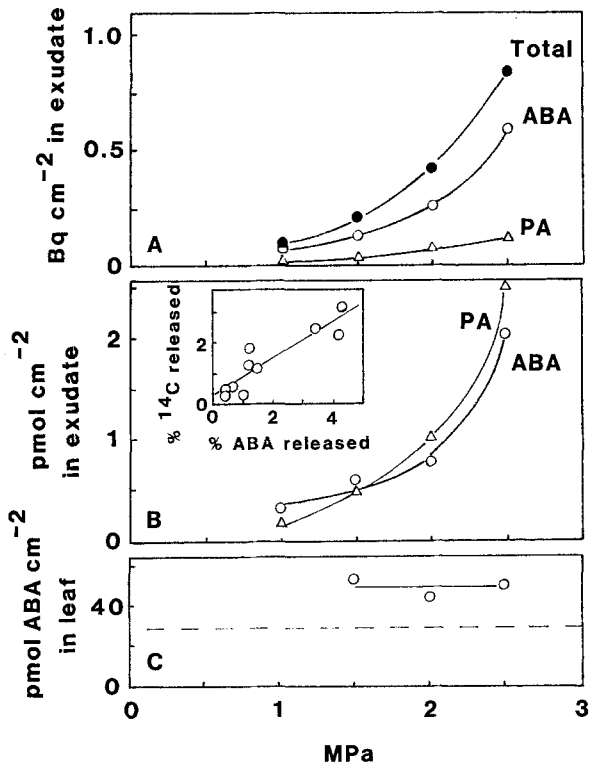

Fig. 3A–C. Release of ^{14}C, ABA, and PA into leaf exudates during pressurization. Excised cotton leaves were preloaded with [^{14}C]ABA in the transpiration stream, then transferred to the pressure chamber. Exudate was collected at 1 MPa until equilibration, then the pressure was increased to either 1.5, 2, or 2.5 MPa and exudate was collected again. After release of the pressure, discs were cut from each blade to determine total radioactivity, which varied between 2.4 and 2.9 kBq in these experiments. The blade was then frozen for later analysis of ABA. **A** Total ^{14}C in exudate, and ^{14}C in exuded solutes. **B** Cumulative amount of ABA or PA in exudate. **C** Total amount of ABA (endogenous plus exogenous) in leaf blade after release of pressure. The *dashed line* indicates the amount of [^{14}C](±) ABA taken up initially. *Insert*: The ^{14}C content of exudates as a function of ABA content when both are expressed as a percentage of the total in the leaf. The regression equation is $Y=0.33+0.59\,x$ ($r=0.90$)

both N levels the old leaves released about twice as much ^{14}C as the young leaves. The differences indicate that both N stress and leaf aging increased the release of ABA into the apoplast. Similarly, water stress before excision increased ^{14}C release at 1.0 MPa to 0.54% (Table 2).

Kinetin had no consistent effect on the release of ^{14}C to the apoplast in leaves from any of the treatments (Table 2). The reversibility of N-stress and water-stress effects on stomata (Table 1) was not reflected in the distributions of ABA with and without kinetin. Indeed, especially in old leaves kinetin tended to increase, rather than decrease, the percentage of radioactivity released (Table 2); however, these differences were not statistically significant (95% probability level). It is apparent that the ABA-kinetin interactions on stomata in N-stressed and water-stressed leaves originated elsewhere.

Table 2. Radioactivity in exudate from excised cotton leaves incubated under pressure. The leaves were preloaded with [^{14}C]ABA with or without 10 μM kinetin in the transpiration stream, then transferred to the pressure chamber and pressurized at 1 MPa. After equilibration at that pressure, the leaves were removed from the chamber and discs were cut from each blade to determine total leaf ^{14}C. Total radioactivity varied between 1.2 and 2.0 kBq in these experiments. Values are means ± SE from three to five experiments. The 95% confidence limits are approximately twice the SE

Leaf age	Stress treatment	Radioactivity in exudate (% of total)	
		− Kinetin	+ Kinetin
Young	Unstressed	0.14±0.11	0.15±0.10
Old	Unstressed	0.29±0.09	0.42±0.14
Young	N stress	0.37±0.03	0.35±0.06
Old	N stress	0.74±0.11	0.93±0.22
Young	Water stress	0.54±0.08	0.49±0.05

One possible explanation of the enhanced ABA release is that cell membrane breakdown at high pressure may have caused non-specific solute release. This possibility was tested by measuring electrical conductivities of exudates. The conductivities of 2-MPa exudates were equivalent to those of 5–10 mM KNO_3 solutions. For comparison, chilling the leaf to near 0° C increased this equivalent concentration to 60 mM, and freezing-thawing increased it to over 200 mM. We conclude that pressurization caused very little membrane damage compared to chilling or freezing.

Discussion

The use of pressure-induced exudation to sample the apoplastic pool of ABA is a new concept that originated with Ackerson (1982) and to our knowledge has been used in only one other laboratory (Cornish and Zeevaart 1985). Ackerson (1982) reported that apoplastic ABA remained at basal levels in rapidly dehydrated leaves which had insufficient time to synthesize ABA de novo. In contrast, Cornish and Zeevaart (1985) found a redistribution of ABA preceding net synthesis by the leaf; their results are consistent with the rapid ABA efflux from stressed leaf tissue reported by Hartung et al. (1983). The data reported here also indicate a redistribution during rapid dehydration. Leaves pressurized to 2.5 MPa released up to 3% of their ^{14}C to the exudate (Fig. 3). As with Hartung et al.

(1983), the use of a [^{14}C]ABA preloading technique precluded any possible contribution of newly synthesized ABA to the measured release.

In addition to specific effects of dehydration on ABA redistribution, two other possibilities could account for the release of label under pressure. First, breakdown of cell membranes could have allowed a non-specific release of solutes. Electrical conductivities of exudates did not support this interpretation. Ackerson (1982) and Cornish and Zeevaart (1985) also discounted membrane breakdown as a cause of ABA release. Second, the more extended incubation in darkness inside the pressure chamber may have increased the release of ABA by altering chloroplast stromal pH (see Cowan et al. 1982). This possibility also seems unlikely because dark incubations of equal length at lower pressures did not similarly increase the release of ABA (compare ^{14}C release at 1.5 and 2.5 MPa in Fig. 3). The phloem, however, as an alkaline compartment of leaves, can "trap" and accumulate large amounts of ABA (Delrot et al. 1981). The degree to which the phloem might contribute to the observed release of ^{14}C (or compete for ABA released from mesophyll cells) was not assessed.

Is enough ABA released to the apoplast to trigger stomatal closure? To answer this question, it was assumed that exogenous (±)-ABA was completely mixed with native (+)-ABA, so that dilution could be estimated. This assumption is supported by considerable evidence that ABA is distributed within cells solely following pH gradients (e.g. Behl et al. 1981) and also by the unchanging specific radioactivity of released ABA with increasing pressure (Fig. 3). It was further assumed that the enantiomeric composition of the ABA in the exudate was equivalent to that in the leaf, i.e., approximately two-thirds (+)-ABA and one-third (−)-ABA. Data in Fig. 3 show that young unstressed leaves released approx. 0.2 pmol·cm^{-2} of (+)-ABA to the 1-MPa exudates and another 1.2 pmol·cm^{-2} between 1 and 2.5 MPa. These amounts can be compared to the dose of 0.8 pmol·cm^2 exogenous (+)-ABA which was earlier deduced to cause incipient (5%) closure in the leaf (Fig. 1). Even with the large additions of [^{14}C]ABA to the tissue (Fig. 3C) (necessary because of its low specific activity and the small percentage released to the exudate), the total (+)-ABA content of the leaf did not exceed the range considered normal for this species. We therefore conclude that ABA is released during dehydration in quantities adequate to initiate stomatal closure. Water-stressed, N-deficient, and old leaves not only released more ^{14}C than young unstressed leaves at a low pressure (Table 2), they also were more sensitive to exogenous ABA (Figs. 1, 2, Table 1). In these cases the ABA released at 1 MPa could well be adequate to affect stomata. Cornish and Zeevaart (1985) reported a similar "aftereffect" of water stress on ABA partitioning in *Xanthium*. Because leaves equilibrated at 1 MPa pressure do not reach zero turgor, the data are consistent with observations of stomatal closure at positive leaf turgor in aged or N-deficient leaves (Jordan et al. 1975; Radin 1981; Radin and Ackerson 1981).

Our results show (1) that leaf dehydration causes the release of ABA from the symplast into the apoplast at a rate large enough to initiate stomatal closure; (2) that this release rate is increased by N stress, prior water stress, and leaf aging; and (3) that N stress, prior water stress, and leaf aging all increase stomatal responsiveness to exogenous ABA. In the case of leaf aging, the partitioning of ABA suffices to explain the enhanced responsiveness. However, kinetin partially blocked the stomatal response to ABA in N-deficient and previously water-stressed leaves despite the increased ABA in the apoplast, indicating that a second factor can also affect stomatal behavior. We conclude that environmental or developmental factors affecting stomatal responses to ABA can do so by two separate mechanisms, one involving altered partitioning between symplast and apoplast, and the other involving some "post-release" site of action. Because cytokinins are effective on stomata in isolated epidermis (Jewer and Incoll 1980), and because they specifically alter the CO_2 feedback loop in ABA-treated tissue (Blackman and Davies 1984a), the ABA-cytokinin interaction presumably occurs at the guard cells.

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